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Factors affecting the accumulation of pentosidine in the skin of wild birds

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ABSTRACT

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Birds have few reliable indicators of aging. Pentosidine (Ps), a biomarker of aging, is a product of non-enzymatic glycation, which accumulates in the tissues of an animal over its lifespan. The intent of this study was to determine if comparable change in Ps concentrations could be established in the skin of wild birds of known ages. Skin samples were obtained from the breast of 44 birds of various species. Foot webbing samples were obtained from 24 California Gulls. Collagen was measured by a hydroxyproline spectrophotometric method and Ps was quantified using reverse phase HPLC. Ps concentration in the skin and foot webbing increased linearly with age ($p<0.001$). Measurement of Ps in birds of unknown ages provided age estimates within the established life-span of these species. Hydroxyproline concentrations from the foot webbing were comparable to that measured in the skin, however Ps concentrations were approximately one-fourth of that in skin. Knowledge regarding the longevity of birds could provide information for species survival programs (SSPs) and insight into variations in longevity of an entire population

Dedication

**This project is dedicated to my mother,
Helene M. Weinberger**

Who by her unwavering and unconditional support and love has allowed me to achieve a positive existence

Acknowledgements

I would like to express my sincere appreciation to my graduate advisor, Dr. Hillar Klandorf, for giving me this opportunity so late in life and for his constant positive encouragement, helpful counsel, and timely assistance throughout the course of this study.

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Section 1 Introduction

Birds exhibit few outward signs of aging. Scientists have struggled with the inability to find reliable methods to predict unknown ages of wild birds. Graying of the muzzle, wear of teeth, wrinkling and coloration of the skin and strength of bones can be used to estimate a mammals age. However, sexually mature birds do not show obvious bodily changes with age.

Bird evolution produced anatomical differences that contribute to the problem of reliable aging. As flight developed, bones became hollow to reduce weight. The original bony jaw and teeth gave way to develop a horn-like beak. Feathers covered the skin to assist in better flight and aid in thermoregulation. The development of these characteristics complicates the ability to quantify aging in birds.

Presently, there are a number of ways to determine the difference between a juvenile and an adult bird. Wildlife biologists, ornithologists and researchers can mark changes in plumage, length of tail-feathers, and spur length (depending on species) to determine adulthood. More invasive measures include checking the bursa a fabricus to determine whether the

bird has reached sexual maturity. While these outward signs provide a fairly reliable method by which to determine adulthood, they do not mark changes past adulthood.

Pentosidine is a protein crosslink that is formed through the nonenzymatic glycation of lysine and arginine residues. Pentosidine is a fluorescent, imidazo pyridinium compound (Sell and Monnier, 1989). Diabetes researchers have utilized biomarkers such as pentosidine to indicate the amount of cross-linked collagen within various tissues. Animals previously studied include rabbits, poultry, rats, and humans (Bellmunt, 1995; Iqbal 1999, Polharna, 1995; Reiser, 1995; Sell,1992).

Pentosidine accumulates exponentially in the collagen of mammals (Sell and Monnier, 1989, Sell et al., 1992). Iqbal confirmed that pentosidine is present in the skin of broiler breeder hens and that, in the hens, as in mammals, its concentrations increase with age (Iqbal et al., 1997,1999).

OBJECTIVE

The Primary objective of this study was to develop an index by which to age birds based on the quantity of pentosidine present in the skin of birds of known ages. This index would provide a convenient method of avian age estimation.

If this aging technique could be applied to living birds, potential applications span various fields. In the case of endangered species, attempts are made to pair the most genetically compatible birds together to ensure best fit offspring. Because many birds mate for life, it is important to mate birds as completely as possible to insure total compatibility. The ability to estimate age in wild birds would eliminate many of the problems associated with pairing of endangered species. In addition, many birds used for research are still wild-caught. The ability to estimate age in these birds will contribute to more accurate data concerning population fluxes, behavioral patterns, and life cycles. Zoo programs (small population management) would also benefit from this technique in the areas of mating, behavioral patterns, and species specific physiology. Medical advances are being made in the study of age-linked diseases in birds. For example, hyperkeratinosis and hemochromotosis have been observed in domestic birds and are known to advance progressively with age. Knowledge of these diseases could aid in understanding of other late-life diseases such as diabetes. Techniques which quantify the accumulation of advanced glycation end-products have unforeseen benefits in other areas of aging research .

Literature review

Aging and birds

Aging is a time dependent biological process common to all multicellular organisms. Aging is associated with a progressive decline in the efficiency of various physiological functions (Sohal and Weindruch, 1996). Aging is defined when two criteria are met: 1) the probability of death at any point in time increases with the age of the organism and 2) characteristic changes in phenotype occur in all individuals over time due to the limiting processes (Johnson et al., 1999). The phenotypic definition is important in distinguishing the aging process itself from diseases of aging. Phenotypes of aging affect all of the individuals in a population, while diseases of aging affect only a subset. There are a variety of strategies, models and hypothesis that have been used to understand the deleterious processes of aging. The consensus among researchers views the concept of aging as a living organism's failure to maintain homeostasis (Holliday, 1992). Aging theories can be distributed into two broad categories based on putative casual forces: 1) intrinsic factors referring to genomic components and genetic processes; and 2) extrinsic factors, encompassing various external influences on the

organism (Iqbal, 1999). Weindruch and co-workers (2001) proposed 5 nonexclusive explanations for the aging process:

1) Damage from oxidative stress (see Sohal *et al.*, 1996)
2) Damage from glycation reactions (see Kristal and Yu 1992)
3) Increases in body temperature associated with a hypermetabolic state (see Walford and Spindler 1997)
4) Alterations in gene and protein expression (see Van Remmen *et al.,* 1995)
5) Neuroendocrine changes (see Nelson *et al.,* 1995)

Weindruch and colleages used microarray technology to survey 6,347 genes from gastrocnemius muscle cells of male mice subjected to caloric restriction. Caloric restriction has been studied extensively as a method to retard the aging process. When compared to the control group, mice subjected to caloric restriction exhibited altered gene expression in 84%(26/31 genes) of the four major gene classes that displayed age-associated alterations (stress, biosynthesis, protein metabolism and energy metabolism) in normal aged mice. Weindruch and co-workers concluded that aging is characterized by an adaptive response consistent with increased production of reactive oxygen species. The Weindruch study proposed that activation of neuronal and myogenic responses to injury were secondary.

In comparison to their mammalian counterparts, birds, in a physiologic sense, age much more slowly. However, among various avian orders there is broad variation in aging rates and patterns. Poultry species (order Galliforms), including domestic chickens, turkeys, cortunix quail, and most common laboratory animals, are the shortest-lived and most rapidly aging birds. Alternatively, parrots (order Psittaciformes), seabirds (Charadriiformes), songbirds (Passeriformes), hummingbirds (Apodiformes), and raptors (Falconiformes) all include representatives with exceptionally long life spans and retarded aging rates for their body size. The comparative longevity and slow aging of birds relative to mammals is documented with data from captive zoo populations and pet birds as well as maximum life-span data from mark-recapture studies of wild birds in nature (Lindstedt and Calder, 1976; Finch, 1990; Holmes and Austed; 1995 Ricklefs, 1998).

The role of free radicals in aging

Relatively higher metabolic rates (Rat=750ul O_2/g/hr., Pigeon=1500ul O_2/g/hr.) require birds to consume more oxygen per gram tissue than mammals of comparable body size (Barja *et al.,* 1994). Damaging reactive

oxygen species (ROS), a by-product of normal metabolism, contribute significantly to the accumulated cellular damage associated with aging.

Holmes et al., (2001) proposed two different mechanisms that could explain how birds minimize oxidative damage that contributes to cellular decline.

1) Birds may produce fewer ROS per mole of oxygen consumed, the result of more efficient mitochondrial electron transfer.
2) Birds may possess increased relative antioxidant activity that provides resistance to damage from ROS.

Two independent studies found H_2O_2 accumulation in tissues of pigeons (C*olombia livia)* was only about one-tenth of that in Norway rats (*Rattus norvegicus*) (Ku and Sohal, 1993; Barja *et al.,* 1994) However, the relative activity of antioxidant enzymes in the two studies was not in agreement. Ku and Sohal (1993) reported an increase in the relative activity of antioxidant enzymes in the pigeon when compared to the rat, whereas Barja and co-workers (1994) found a general decrease in the antioxidant enzyme (Superoxide dismutase, Catalase, Selenium-dependent GSH-peroxidase, GSH-reductase, and Ascorbate) activity of pigeons compared to rats. Herrero and Barja (1988) also found lower

antioxidant enzyme activity in adult male parakeets (Melopsittacus undulatus) and canaries (Serinus canarius) when compared to rats.

Ogburn and colleagues (1998) determined that avian cells have a greater resistance to oxidative challenge when cultured kidney epithelial cells were exposed to various pro-oxidants (95% oxygen, H_2O_2, and paraquat). The results of the Ogburn study showed that cells from three long-lived bird species survived longer and suffered less DNA damage than did mouse cells.

The defenses that birds possess with regard to oxidative stress could be structural and constituitive (decreased ROS production per mole of oxygen) or inducible (increased relative antioxidant activity) but more likely are a combination of both.

The Maillard reaction

The Maillard reaction is the non-enzymatic attachment of reducing sugars with amino groups on protein. The reaction is initiated when an aldehyde group (CHO) of glucose and an amino group (NH_2) of protein combine. This formation is termed a Schiff base and is very unstable. The Schiff base quickly rearranges itself into a more stable substance known as an Amadori product (Cerami *et al*., 1987). These Amadori products slowly dehydrate and rearrange themselves into irreversible structures termed advanced

glycation end products (AGE's). Many AGE's are able to cross-link with adjacent proteins. Maillard reactions have been targeted as a key player in age-related diseases and diabetic pathological conditions (Cerami, 1985).

Advanced glycation end-products

Advanced glycation end-products have been used as biomarkers of aging in tissues of humans and laboratory animals. AGE's are thought to cause the age-dependent yellowing and crosslinking in long lived proteins such as lens crystallins and collagen. Cellular receptors recognize AGE's and can initiate cellular events that induce tissue damage associated with aging. Kristal and Yu (1992) developed the synergistic theory of aging based on the observation that AGE's require successive oxidative steps which may be associated with free radical damage. The structures of AGE's that have been elucidated include carboxymethllysine (CML), pentosidine (Ps), and arginine-lysine imidizole (ALI) (Wells-Knecht *et al.,*1996, See Fig 1). Chellan and Nagaraj (1999) established the first HPLC technique to quantify the dicarbonyl derived imidazoliam crosslinks methylglyoxal-lysine-dimer (MOLD) and glyoxal-lysine-dimer (GOLD). GOLD and MOLD are neither chromophores nor flourophores which prompted Chellan and Nagaraj to use their phenylisothiocyanate (PITC) derivatives for UV detection. AGE's have

now been measured in a small number of bird species including glycated hemoglobin studies in the long-lived avian order Apodiformes (hummingbirds) (Beuchat and Chong, 1998) as well as pentosidine concentration in skin collagen in the short-lived order Galliformes (domestic chickens) (Iqbal *et al*., 1999, Klandorf *et al*., 1999)

Pentosidine as a biomarker for aging

Pentosidine is a widely documented Maillard structure first islolated by Sell and Monnier (1989a) and described as an imidazopyridinium compound (Sell and Monnier, 1989b) comprised of single lysine and arginine moieties crosslinked by a pentose. It was identified as the product of the Maillard reaction elicited by hexoses, pentoses, ascorbate and a variety of Amadori compounds *in vitro* (Sell and Monnier 1989a, 1989b; Dyer *et al*., 1991; Grandhee and Monnier, 1991). However, the major *in vivo* carbohydrate source leading to the formation of pentosidine is not known.

Oxidation reactions are required at some stage of the reaction as pentosidine formation is inhibited in the absence of oxygen. Baynes (1991) introduced the phrase ‘glycoxidation product’ to describe pentosidine and other glycation compounds similarly affected by the availability of oxygen. Pentosidine has been found to increase exponentially in the collagen of

mammals over the animal's lifespan (Sell and Monnier, 1989, Sell et al., 1992). Iqbal confirmed that pentosidine is present in the skin of broiler breeder hens and that, in the hens, as in mammals, it accumulates with age (Iqbal et al., 1997,1999). Diabetes researchers have utilized biomarkers such as pentosidine to indicate the amount of cross-linked collagen within various human tissues. The measurement of pentosidine in the tissues of animals is greatly facilitated by its acid stability and its flourescent properties. However, Dyer *et al.,* (1991) presented evidence that pentosidine represents less than 1% of the total crosslinks formed during the *in vitro* Maillard reaction of proteins with glucose. Fast atom bombardment high resolution mass spectrometry showed a mass/charge of 379, 2069 compatible with the empirical formula $C_{12}H_{27}N_60_4$.

Collagen crosslinking

Collagen is a ubiquitous tissue protein that constitutes approximately 30% of the protein in mammals. Collagen has been identified in 19 different forms and has a variety of biological functions (McCormick, 1999).

The physical properties of collagen change as a result of cross-linking and contribute to the stiffness in skin, tendon, joints, and overall tissue rigidity. The accumulation of AGE's is associated with alterations in

mechanical properties, solubility, ligand binding and conformation of collagen (Reiser, 1991). Collagen can be separated by type and location. Type I, II, and III collagen are the most abundant types with type I collagen accounting for 90% of total body collagen. Type I,II, and III collagen are long fibrous rod-like molecules. Type I collagen forms the connective tissue of skin, bone, tendon, ligaments, dentine, sclera, fascia, and organ capsules. Type II collagen is a major component of cartilage, notochord, and the intervertebral disk. Type III collagen is associated with the connective tissue of organs (uterus, liver, spleen, kidney, lung, etc.) smooth muscle, endoneurium, blood vessels, and fetal skin (Ross et al., 1995). Collagen crosslinks can be classified into two major areas:

1.) Crosslinks that are important during normal development and maturation and are controlled by the enzyme lysyl oxidase (Siegel 1979; Reiser et al., 1992)

2.) Crosslinks that are derived from the non-enzymatic addition of carbohydrate moieties and contribute to accelerated tissue aging (Cerami 1985; Baynes and Monnier, 1989; Reiser, 1991)

Recent theories suggest that non-enzymatic glycosylation of tissue proteins in combination with other processes such as free radical generation contribute significantly to the aging process (Masoro *et al.,* 1989; Kristal and Yu, 1992; Weindruch *et al.,* 2001). Verzigl and colleagues (2000) calculated the half-life of human cartilage collagen to be 117 years and that of skin collagen to be 15 years. Collagen molecules provide a reliable method by which to quantify pentosidine crosslinks and other AGE's due to their exceptionally long half-life.

Aging in Birds Vs. Mammals

Birds are remarkably longer–lived than mammals of comparable body size. Maximum longevity of wild birds average 1.7 times greater than captive mammals, and captive birds are nearly three times as long –lived as captive mammals (Lindstedt and Calder, 1976; Austed and Fischer, 1991). Scarlet Macaws (*Ara macao*) have documented captive life spans to over 90 years. This is more than four times longer than would be predicted for a mammal of equivalent body size. Common Ravens, (*Corvus corax),* have lived in captivity up to 69 years. Hummingbirds are the most disproportionately long-lived birds. Hummingbirds are the smallest bird species with the highest metabolic rate (Holmes and Austed, 1995). The Planalto Hermit,

(*Phaethirnis pretrei),* a hummingbird with an average weight of 15 grams, has a recorded captive lifespan of 14 years. The shortest lived and most rapidly producing and senescing bird species of any size documented to date is the Common Quail, Coturnix, with a mean life span of one year in the wild (Holmes and Austed, 1985). However, this 90g bird ages much more slowly than an equivalent sized rodent.

A deeply rooted theory of aging suggests that an organism's longevity is inversely related to its metabolic rate (Comfort, 1979; Holliday, 1992). The validity of the metabolic rate hypothesis has been challenged due to recent studies with caloric restricted (CR) animals. Caloric restriction has long been known to extend the life span of rodents (Weindruch and Walford, 1982, 1988). However, Masoro and co-workers (1991) found that caloric restricted rats have metabolic rates similar to their non-restricted counterparts, yet have much longer lifespans. Holmes and Austed (1995) called for a reassessment of metabolic rate as the major determinant in the aging process based on substantial evidence from comparative studies of various species of birds. The results from these collective studies find that birds and bats have substantially longer life spans than would be predicted based on their metabolic rate (Holmes and Austed, 1995; Barja et al., 1994). Consideration must also be given to how selection pressure will effect aging.

Namely, other things being equal, species subject to low levels of extrinsic mortality can age more slowly than species subject to high mortality rates. This theory of aging predicts that most birds should age more slowly than mammals because flight affords protection against extrinsic mortality due to predation or accident (Edney and Gill, 1967; Austed and Fischer, 1991).

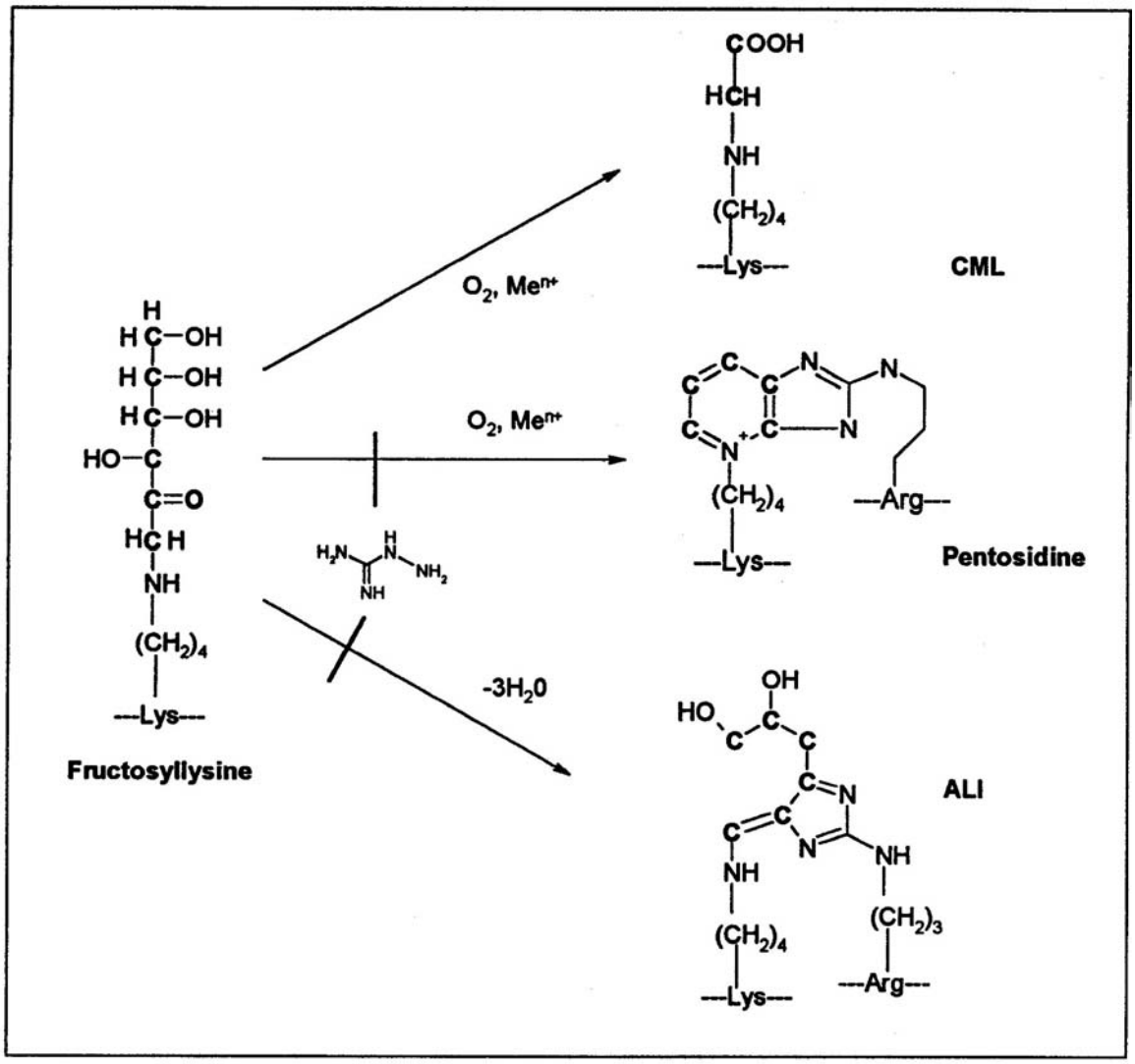
Birds as animal models for aging

The longevity of birds provides researchers with an interesting paradigm because birds exhibit several traits which current theories of aging suggest should render them more susceptible the degenerative processes of aging.

Birds have metabolic rates as much as 2.0-2.5 times higher than those of similar sized mammals (Lindstedt and Caulde, 1976); concentrations of plasma glucose typically 2.0-6.0 times higher than those of mammals, and a body temperature approximately 3° Celsius higher (Holmes and Austad, 1995). Modern theories of aging consider an increase in any one of these parameters as contributors to accelerated tissue damage due to the accumulation of deleterious by-products of oxidative metabolism and the Maillard reaction (Cerami, 1985; Monnier *et al.,* 1991; Kristal and Yu, 1992; Weindruch *et al.,* 2001). Birds seem to have developed mechanisms to protect against free radical damage and advanced Maillard reactions,

consequences of high metabolism, high body temperature, and elevated blood glucose. It has been proposed that birds have special mechanisms to prevent the formation of such products, degrade them once they are formed, remove affected cells, or some combination of the three to live longer (Holmes and Austed, 1995; Monnier, 1990; Iqbal *et al.,* 1997). Class aves offers a unique perspective from which to study the proposed protective mechanisms that limit free radical production, Maillard reaction damage, AGEs formation, and may offer insight into medical intervention for retarding aging.

Figure 1. Structure and pathways of formation for three advanced glycation end-products (AGEs). Formation of carboxymethyllysine (CML) and pentosidine involves oxidative steps while arginine-lysine imidizole (ALI) forms non-oxidatively.


COOH
HCH
NH
(CH2)4
Lys
CML
O2, Men+
O2, Men+
Arg
Lys
Pentosidine
-3H20
OH
HO
ALI
NH
NH
(CH2)3
(CH2)4
Lys
Arg
H
HC–OH
HC–OH
HC–OH
HO–C
C=O
HCH
NH
(CH2)4
Lys
Fructosyllysine

Pentosidine as a measurement of chronological age in wild birds

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In many species, visual clues can be used to help estimate age, from skin wrinkling in humans to graying or whitening of the muzzle and wear of teeth in many other mammalian species. However, in birds, evolution has produced anatomical differences that contribute to the problem of reliable aging. The original bony jaw and teeth gave way to development of a horn-like beak. Feathers covered the skin to assist in better flight and aid in thermoregulation. The development of these characteristics complicates the ability to quantify aging in birds.

Pentosidine is a product of non-enzymatic glycation (the attachment of a carbohydrate moiety to lysine and arginine residues without the aid of enzymes) and it has been described as a fluorescent, imidazo pyridinium compound (Sell and Monnier, 1989). Collagen pentosidine has been found to increase exponentially over a mammals lifespan. (Sell and Monnier, 1989, Sell et al., 1992). Iqbal confirmed that pentosidine is present in the skin of broiler breeder hens and that, in hens, as in mammals, its concentrations increase with age (Iqbal et al., 1997,1999). Diabetes researchers have utilized biomarkers such as pentosidine to measure cross-linked collagen

within various tissues. Numerous studies with mammals and domestic poultry have validated the use of pentosidine as a biomarker for aging (Bellmunt, 1995; Iqbal 1999, Polharna, 1995; Reiser, 1995; Sell,1992).

We are using pentosidine to estimate the age of wild birds. Reliable estimation of a bird's age could play a critical role in Species Survival Plans (SSPs) and the pairing of endangered species. Many birds, especially passerines, are still wild-caught. If this technique could be applied to living birds, it will help us gain insight concerning life cycle and population fluxes (i.e. maximum and average life span, generational span, onset and length of reproductive life, etc).

By compiling pentosidine data from a variety of species and ages we were able estimate the ages of unknown birds. Additionally, continuing data collection will permit the correlation of pentosidine accumulation as a function of habitat, diet, and migratory status.

Materials and Methods

To determine the relationship between pentosidine accumulation and age we obtained approximately 20 grams of skin from the lateral sides of the abdomen and thorax of previously frozen birds. Samples were obtained in collaboration with James Bonner, Curator of Birds, National Aviary,

Pittsburgh, PA. Samples were stored at –70 degrees C. The samples collected consisted of 46 birds of known ages and 5 birds of unknown ages spanning 32 different species. The ages of these birds varied from a few days to 18.5 years. A second study was conducted using foot webbing from California Gulls of known ages (n=17). The samples were obtained as part of a blind study in cooperation with Donna Holmes from the University of Idaho and Bruce Pugesek from the University of Washington. Footweb skin specimens were collected in July and August, 1997, from members (adults and chicks near fledging age) of a breeding colony of California gulls (*Larus californicus*) located on an island in Bamforth Lake, Adams County, Wyoming. Tissue samples were collected within 30 min. from the time of death. Skin was excised (about 1.5 to 2 cm in area) from between the toes of each bird. Skin was immediately placed into sterile saline and quick-frozen on dry ice. Samples were subsequently stored at –70 degrees C. Specimens were coded so that ages and identities of birds were unknown to our lab during the pentosidine quantification procedure. The samples of foot webbing and breast skin were prepared and quantified similarly.

The skin was prepared according to the techniques described by Monnier *et al.,* (1986), Sell *et al.,* (1992), and Iqbal *et al.,* (1997). Briefly, this technique involved the removal of the epidermal and adipose layers

from the skin, homogenization, and extraction with 2:1 chloroform – methanol. Further preparation of the digest for the pentosidine assay involves digestion with 6N HCl, purging with N_2 , heating for 18 hours at 110°C, evaporating the acid in a centrifuge-type vacuum drier (Savant Instruments, Farmingdale, NY), reconstituting in 500ul water and final filtration using a .45 micron Costar Spin-X centrifuge tube filter (Corning Costar Corp., Cambridge, MA) A modified Stegman and Stadler spectrophotometric method for hydroxyproline was used for estimation of collagen in the reconstituted digest. The assumption was made that collagen was 14% hydroxyproline by weight (Maekawa *et al.* 1970). Pentosidine concentration was estimated using a reverse-phase HPLC procedure. Thirty-five microliters of sample were injected into a Waters 2690 HPLC (Waters Corporation, Milford, Mass) with an on line scanning florescence detector (Waters 494, excitation 325 nm, emission 370 nm) used to monitor the pentosidine peaks. Duplicate samples were spiked with a known concentration of pentosidine to ensure quantification of the correct peak (see fig. 1). Separations were achieved by the application of a linear gradient of 5-28% acetonitrile from 0 to 35 min in water and 0.01M heptafluorobutyric acid at a flow rate of 1ml/min. Final quantification of pentosidine was made by comparing peak areas with a pentosidine standard (Vincent M. Monnier,

Case Western University, Cleveland, OH) curve obtained under similar conditions (see fig. 4). The software package Millennium 32 Version 3.05.01 was used for the integration of peaks.

Optimization of HPLC method

HPLC technique for quantification of bird skin pentosidine was modified from a method described by Iqbal and co-workers in 1999. The linear gradient of acetonitrile was reduced from 12-42% to 5-28%. The gradient run was lengthened in time from 20 to 35 minutes. Sharper peaks were obtained using a C-18 column (YMC ODS-AQ 4.6 x 250mm) with a 5um particle size. The column was cleaned for 18 minutes between each run and allowed to equilibrate for 12 minutes. Utilization of this technique provided greater separation from unknown peaks with minimal tailing and consistent retention times.

Statistical Analyses

Regression analysis was performed to determine the correlation between the concentration of pentosidine and chronological age. Data was analyzed using JMP statistical software (SAS Institute Inc., Cary, N.C.) A two-tailed t-test was performed to determine differences in the mean

collagen content and the mean pentosidine concentration in breast skin and foot webbing.

Results

Pentosidine was found in the skin of various species of wild birds and its concentration increased linearly with age ($P<0.001$) (see fig. 2). Linear regression analysis provided the best fit ($y=0.2047x+7.4725$, $R^2 = 0.73$) for breast skin pentosidine concentration as a function of age. Alternative regression analysis did not improve the correlation between breast skin pentosidine concentration and age. In addition, given the strong correlation between pentosidine accumulation and age, we have estimated the ages of birds with previously unknown ages (see Table 1). All age estimations are within the range of each species maximum life span. Pentosidine concentration accumulated in a linear fashion with age in foot webbing. ($P<0.001$) (see fig. 3). Linear regression analysis provided the best fit ($y=0.013x+3.8467$, $R^2=0.70$) for foot webbing pentosidine concentration as a function of age. Alternative regression analysis did not improve the correlation between foot webbing pentosidine concentrations and age. Different areas of the foot webbing from the same sample showed no difference in pentosidine concentration (see sample A= 4.78pmol Ps/mg

collagen vs. sample AM =4.95pmol Ps/mg collagen, N=1, Table 3). The mean collagen content and mean pentosidine concentrations were calculated for breast skin and foot webbing. Samples from the breast skin were age selected to reflect the ages of the birds from which the foot webbing samples were obtained (48-288 months). The mean collagen content in breast skin (5.73±3.04mg/collagen/40mg tissue) was not significantly different when compared to the mean collagen content of foot webbing (3.63±.963mg/collagen/40mg tissue). However, the mean pentosidine concentration in breast skin (18.9±13.7pmol Ps/mg collagen) was noticeably higher when compared to the mean pentosidine concentration in foot webbing (5.72±1.88 pmol Ps/mg collagen) although statistical analysis with a two-tailed t-test showed no difference between the means (see Table 2).

Discussion

Numerous species of birds, including parrots (order Psittaciformes), seabirds (Charadriiformes), songbirds (Passeriformes), hummingbirds (Apodiformes), and raptors (Falconiformes) live for unusually long periods of time when compared to mammals of comparable body size (Lindstedt and Calder, 1976; Finch, 1990; Holmes and Austed, 1995 Ricklefs, 1998). Poultry species (order Galliforms), including domestic chickens, turkeys,

and cortunix quail, are the shortest-lived and most rapidly aging of bird species.

Advanced glycation endproducts (AGEs) have been measured in a small number of bird species including glycated hemoglobin studies using boronate affinity chromatography (Beuchat and Chong, 1998). Beuchat and Chong reported levels of glycohemoglobin to be lower in duck, chicken, and turkey (0.5-1.0 percent range) when compared to the average values for mammals (1.7-5.8 percent range). However, bird erythrocytes are less permeable to glucose than most mammalian red blood cells and also have half-lives of 50-70 percent of mammalian cells. Pentosidine concentration in skin collagen has been measured in the short-lived order Galliformes (domestic chickens) and its concentration shown to increase linearly with age (Iqbal *et al*., 1999, Klandorf *et al*., 1999) . Pentosidine detection in the tissues of animals is greatly facilitated by its acid stability and its flourescent properties. Collagen molecules provide a reliable method by which to quantify pentosidine crosslinks and other AGE's due to their exceptionally long half-life (117 years in cartilage collagen, 15 years in skin collagen) (Verzigl et al., 2000).

The idea to estimate chronological age of avian species via analysis of pentosidine concentration in skin is unique. The results of this study

demonstrate that not only is pentosidine present in the skin of various species of wild birds, but that it can be used as a reliable estimator of chronological age. Pentosidine concentrations had greater variability as the ages of the birds increased. Differences in metabolism, diet, habitat, and sex could play a role in pentosidine accumulation from species to species. However, a strong correlation between pentosidine accumulation and age still exists when these factors are not represented in the model. In order to determine the effect of metabolism, diet, habitat, and sex on pentosidine accumulation, further data collection will be necessary.

Potential nonexclusive explanations for the slightly lower concentration of pentosidine in the foot webbing include:

1) Low vascularization to the tissue, resulting inlimited flow of glucose residues necessary to form Amadori products,

2) lower tissue temperature retards the rate of the non-enzymatic reaction,

3) higher levels of uric acid in the extremities (due to lower temperatures) limit the generation of glycoxidation products. Uric acid is highly insoluble in plasma and readily precipitates at higher concentrations.

The strong correlation between pentosidine concentration and age in foot webbing indicate that a non-lethal technique using biopsy punches from the bird foot may be established. This procedure for age estimation will be of

great benefit to wildlife biologists and researchers in the field of wildlife biology.

The longevity of birds provides researchers with an interesting paradigm because they exhibit several traits which current theories of aging suggest should render them more susceptible the degenerative processes of aging. Birds have metabolic rates as much as 2.0-2.5 times higher than those of similar sized mammals (Lindstedt and Caulde, 1976); concentrations of plasma glucose typically 2.0-6.0 times higher than those of mammals, and a body temperature approximately 3° Celsius higher (Holmes and Austad, 1995). Modern theories of aging consider elevation of these parameters contributors to accelerated tissue damage from the accumulation of deleterious by-products of oxidative metabolism and the Maillard reaction (Cerami, 1985; Monnier *et al.,* 1991; Kristal and Yu, 1992; Weindruch *et al.,* 2001). Birds seem to have developed mechanisms to protect against free radical damage and advanced Maillard reactions, consequences of high metabolism, high body temperature, and elevated blood glucose. It has been proposed that birds may have special mechanisms to prevent the formation of such products, degrade them once they are formed, remove affected cells, or some combination of the three to live longer (Holmes and Austed, 1995; Monnier, 1990; Iqbal *et al.,* 1997). Class aves offers a unique perspective

from which to study the proposed protective mechanisms that limit free radical production, Maillard reaction damage, AGEs formation, and may offer insight into medical intervention for retarding aging.

The ability to establish a technique for age estimation in wild birds will provide far-reaching benefits to aviculture, ornithology, and science in general. Such information could have a significant impact not only on the database of general avian knowledge, but also on the survival of the most critically endangered species.

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Figure 1 A) Pentosidine standard injected under identical conditions as B) Biological sample of an eight and one-half year old Lilac roller. C) Biological sample of a three year-old Red siskin D) Biological sample of three year-old Red siskin spiked with a known concentration of pentosidine.

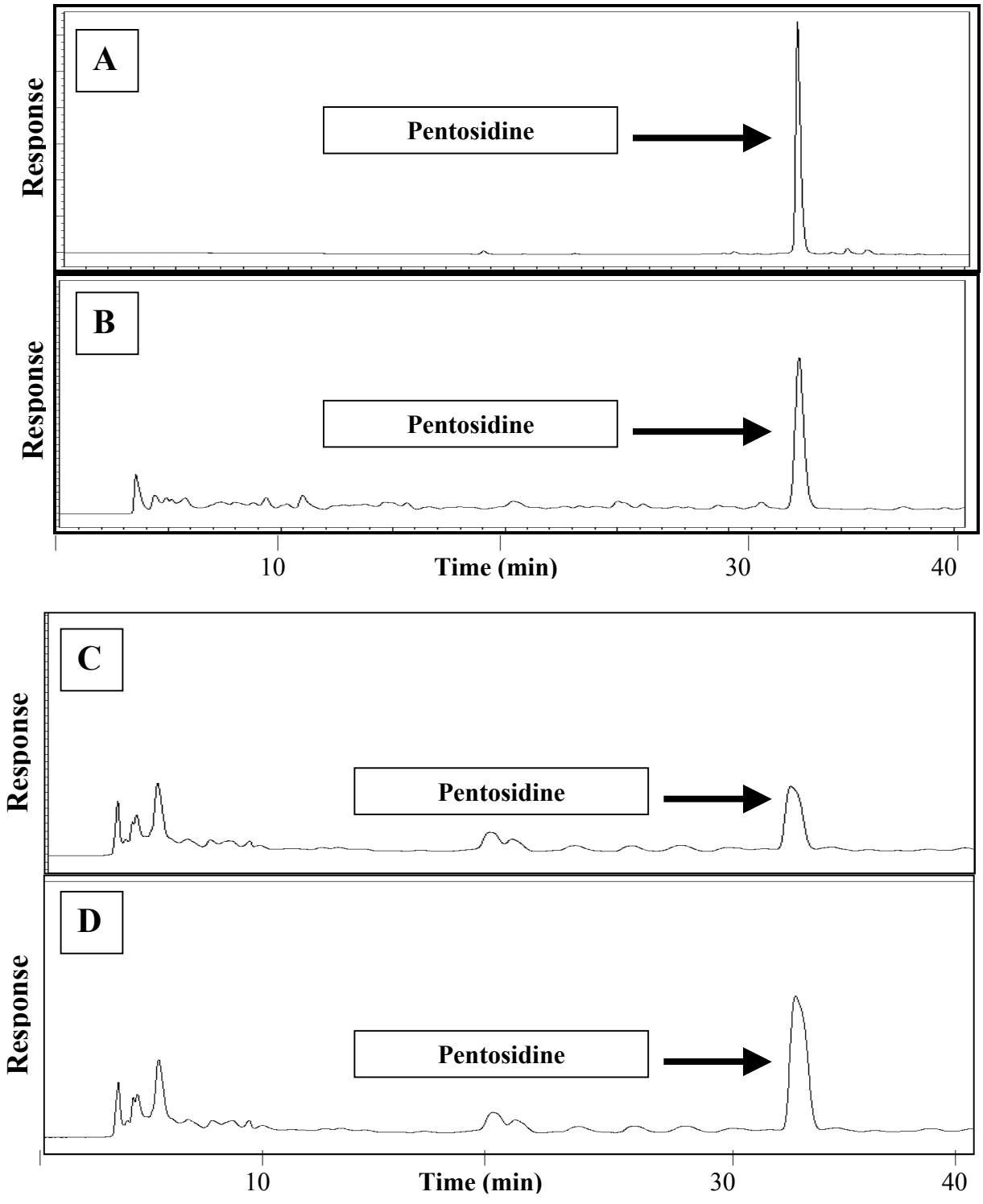

A
Pentosidine
Response
B
Pentosidine
Response
10
Time (min)
30
40
C
Pentosidine
Response
D
Pentosidine
Response
10
Time (min)
30
40

Figure 2 Pentosidine concentration in the breast skin of wild birds as a function of chronological age.

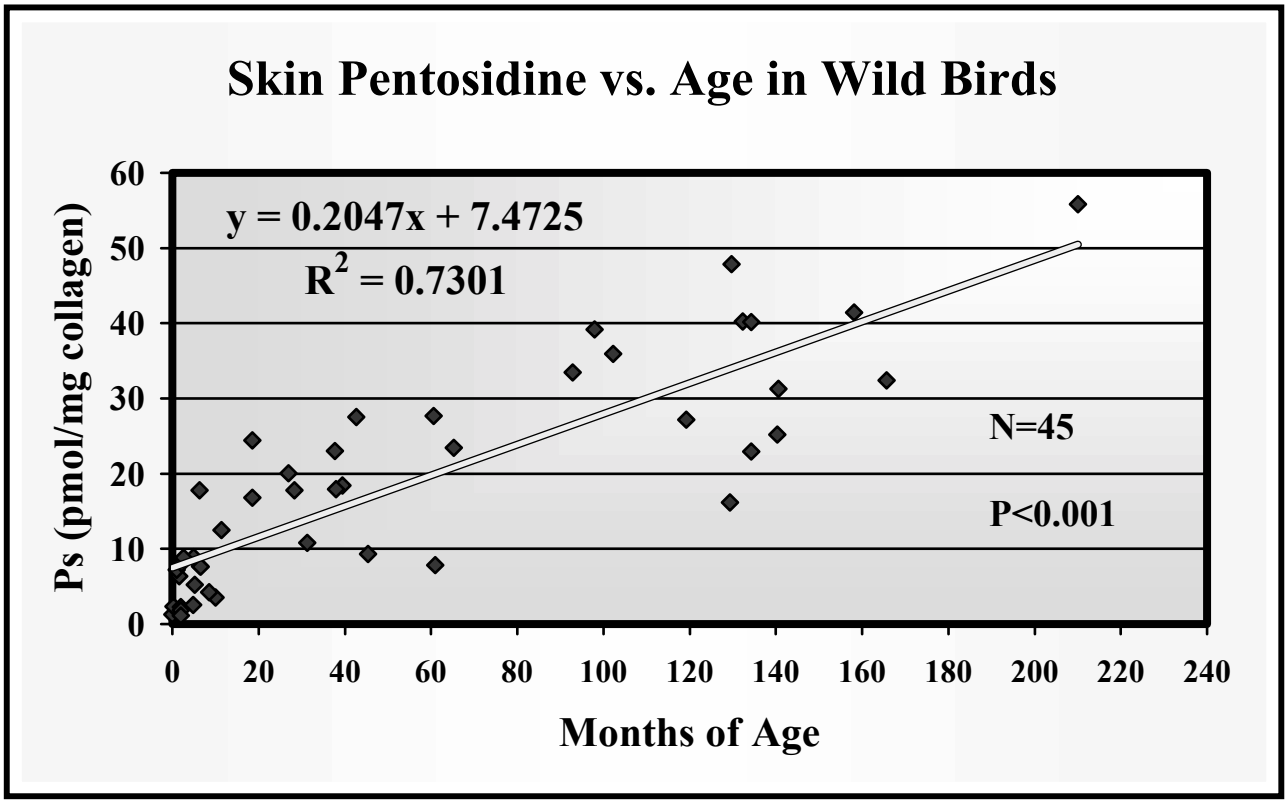

Skin Pentosidine vs. Age in Wild Birds
y = 0.2047x + 7.4725
R2 = 0.7301
N=45
P<0.001
Ps (pmol/mg collagen)
Months of Age
0
10
20
30
40
50
60
0
20
40
60
80
100
120
140
160
180
200
220
240

Figure 3 Pentosidine concentration in the foot webbing as a function of age in California gulls.

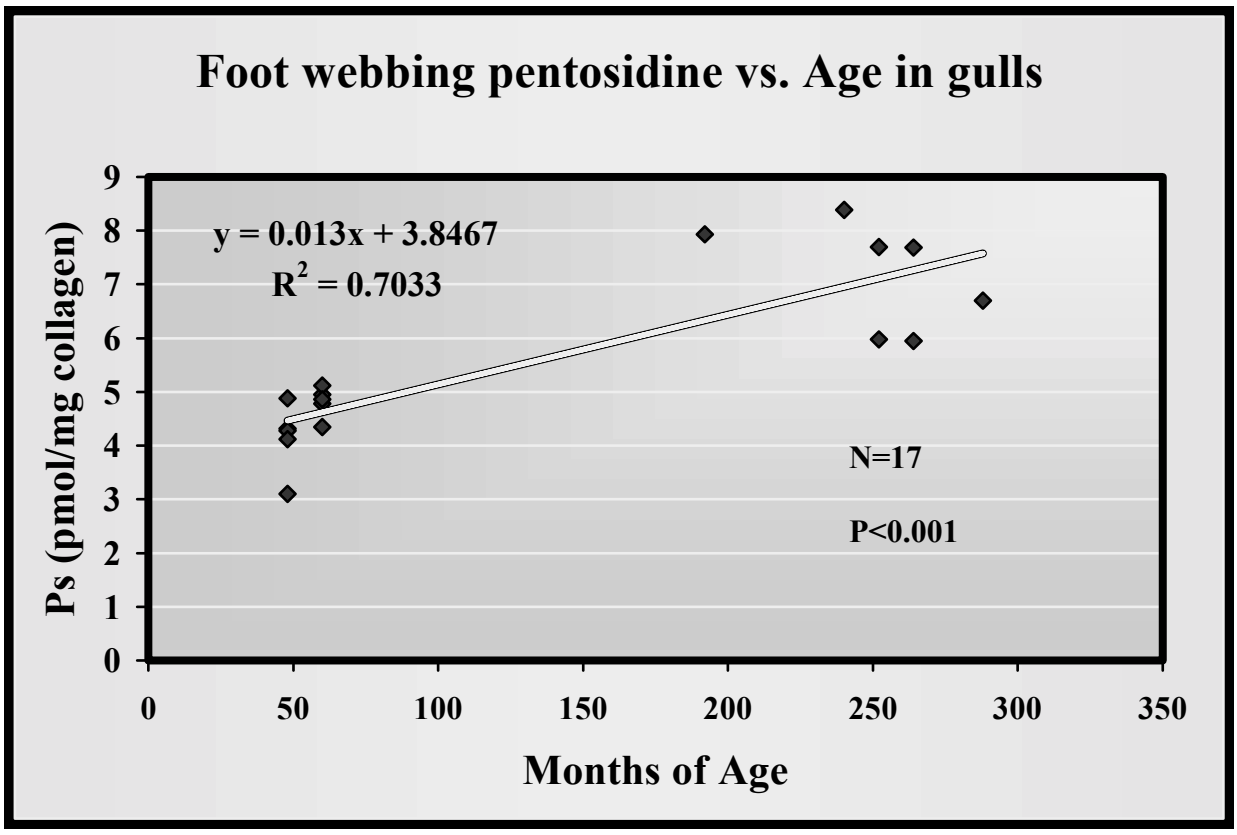

Foot webbing pentosidine vs. Age in gulls
y = 0.013x + 3.8467
R2 = 0.7033
N=17
P<0.001
Ps (pmol/mg collagen)
Months of Age
0
1
2
3
4
5
6
7
8
9
0
50
100
150
200
250
300
350

Table 1 Estimation of age for birds of unknown ages using pentosidine concentration data compiled from birds of known ages.

Common Name	Pentosidine pm/mg collagen	Age in Months
Rock Dove	14.96	36.58
Ring-Necked Pheasant	25.68	88.95
Racing Pigeon	27.13	96.03
Golden-Hooded Tanager	28.72	103.80
Boat-Billed Heron	59.55	254.41

Table 2 Mean collagen and pentosidine concentrations in breast skin vs. foot webbing. No significant differences between means with two-tailed t-test.

Sample	mg/collagen/40mg tissue	pmol Ps/mg collagen
Breast skin	**5.73 ± 3.04**	**18.90 ± 13.70**
Foot webbing	**3.63 ± .963**	**5.72 ± 1.88**

Table 3 List of all California Gull samples. Table includes areas from HPLC analysis, pentosidine concentration and age

GULL SAMPLES 2/28-3/2 (2000)								
Sample #	Avg Area	Avg Area/ul injected	#ul=1mg Collagen	Total Area=1mg Collagen	**a (standards)**	**b (standards)**	**Ps=pmol/mg collagen**	**Age(Months)**
AM	1649127	25767.61	**54.052**	1392790.822	**283628**	**11561**	4.95	60.00
A	1465410	22897.03	**58.663**	1343208.544	**283628**	**11561**	4.78	60.00
B	1152820	18012.81	**76.841**	1384122.525	**283628**	**11561**	4.92	276+
C	1329198	20768.72	**58.506**	1215094.659	**283628**	**11561**	4.32	48.00
D	1085915	16967.42	**80.917**	1372952.876	**283628**	**11561**	4.88	48.00
E	1635296	25551.50	**87.817**	2243856.076	**283628**	**11561**	7.95	unk
F	976168	15252.63	**76.699**	1169861.085	**283628**	**11561**	4.17	unk
G	1593102	24892.22	**67.343**	1676316.687	**283628**	**11561**	5.95	264.00
H	1955675	30557.42	**71.021**	2170218.659	**283628**	**11561**	7.69	264.00
I	1306344	20411.63	**82.558**	1685142.937	**283628**	**11561**	5.98	252.00
J	1664274	26004.28	**86.022**	2236940.282	**283628**	**11561**	7.93	192.00
K	1173329	18333.27	**78.538**	1439858.016	**283628**	**11561**	5.12	60.00
L	1072781	16762.20	**72.948**	1222769.194	**283628**	**11561**	4.35	60.00
M	1765428	27584.81	**83.459**	2302200.866	**283628**	**11561**	8.16	unk
N	1351115	21111.17	**64.697**	1365829.487	**283628**	**11561**	4.86	60.00
O	1422187	22221.67	**54.159**	1203503.527	**283628**	**11561**	4.28	48.00
P	1387682	21682.53	**126.916**	2751860.136	**283628**	**11561**	9.74	264+
Q	1477833	23091.14	**37.524**	866471.9608	**283628**	**11561**	3.10	48.00
R	637630	9962.97	**100.444**	1000720.433	**283628**	**11561**	3.57	unk
S	758897	11857.77	**62.515**	741288.218	**283628**	**11561**	2.65	unk
T	1584805	24762.58	**95.636**	2368193.922	**283628**	**11561**	8.39	240.00
U	1619659	25307.17	**74.629**	1888648.93	**283628**	**11561**	6.70	288.00
V	2479856	38747.75	**56.058**	2172121.37	**283628**	**11561**	7.70	252.00
W	1390374	21724.59	**53.301**	1157942.571	**283628**	**11561**	4.12	48.00
X	2093971	32718.30	**58.838**	1925079.152	**283628**	**11561**	6.83	unk

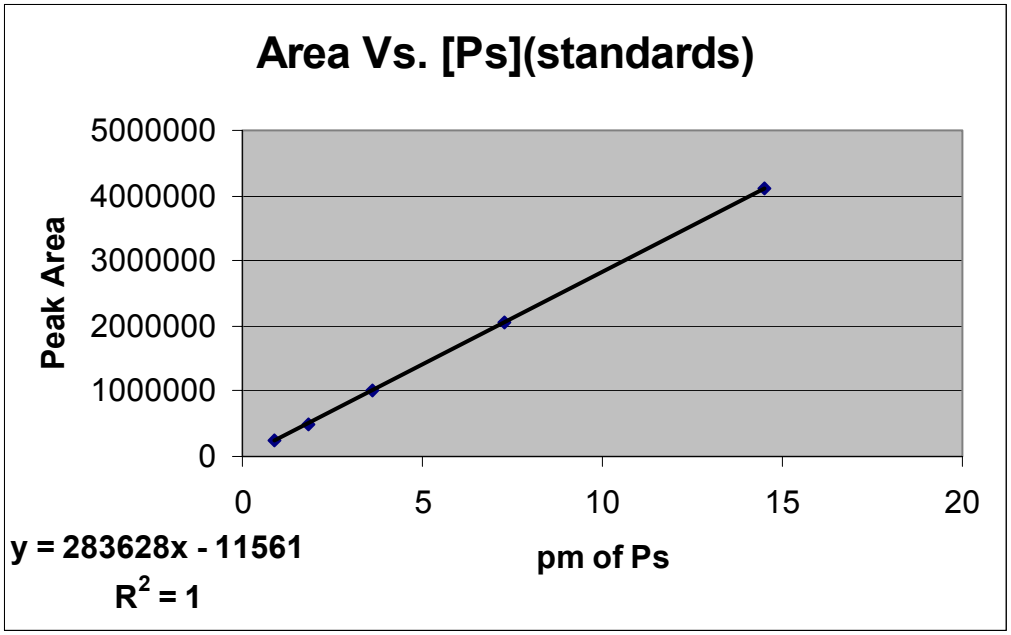
Table 4 List of all wild bird samples including diet, Bw, genus, species and HPLC areas with pentosidine concentration and age.

P3	3149	Snowy owl	1710	f	C	Nyctea	scandiaca	44.84	9917122	41.32	158.2
P4	3259	Fairy blue bird	81.9	m	F,O	Irena	puella	*2231.82	239392	81.37	121.23
Aviary Data #3 Wednsday May 26th, 1999											
S.NO	ISIS	COMMON NAME	BW(G)	SEX	DIET	GENUS	SPECIES	#uL=1mg Coll	Ps Area	Ps=pm/mg collagen	Age in Months
1	2753	Golden-breasted Starling	56.1	male	O	Cosmopsarus	regius	74.68	5784136	40.14	132.33
2	3040	Black necked stilt	165	male	O	Himantopus	himantopus	*108.89	1590271.5	16.13	129.93
3	4710	Red-throated bee-eater	23	unk	N,I	Merops	bulocki	196.74	1096778	20.08	26.93
4	5267	Amethyst starling	39.3	female	O	Cinnyricinclus	leucogaster	72.81	1305712	8.88	5
5	4866	Golden-headed manakin	12.56	female	F,O	Pipra	erythrocephala	287.76	911159	24.39	18.5
6	5212	Quail finch	10.6	unk	G	Ortygospiza	unk. Sp.	*897.63	324076	27.11	4.8
7	4477	Burnished-buffed Tananger	20.7	male	O	Tangara	cayana	319.88	922928	27.45	42.7
8	1977	Many-colored chaco finch	27.2	male	O	Saltatricula	multicolor	~287.11	1209902	32.37	165.63
9	5201	Parson's finch	17.9	unk	G	Poephila	cincta	154.76	439543	6.34	1.66
10	5283	Golden-hooded tananger	9.83	unk	O	Tangara	larvata	453.92	170945	7.23	1
11	1099	Golden-hooded tananger			O	Tangara	larvata	272.08	1132638	28.72	unk
12	4990	Racing Pigeon	298	unk	G,O	Columba	livia	*64.20	6875595.5	41.18	13.03
13	5023	Golden-headed Manakin	13.58	male	O	Pipra	erythrocephala	~171.96	1014421.5	12.47	11.4
14	5282	Chilean Tinamou		unk	H,O	Nothoprocta	perdicaria	393.76	34378	1.26	0.03
15	5236	Red Siskin	9.4	male	G	Cardelis	cucullata	101.49	1948311	18.43	39.4
16	5291	No Name Pheasant Pigeon	unk	unk	O	Otidiphaps	nobilis	~178.42	181124	2.31	0.233
17	4866	Golden-headed Manakin	12.56	female	O	Pipra	etythrocephala	118.86	1514904.5	16.78	18.5
18	3122	Boat-billed Heron	589	male	P,O	Cochlearius	cochlearius	80.75	7912601	59.55	119.83
19	2172	Hyacynth Macaw	1559	female	O	Anodorhynchus	hyacinthinus	*48.98	6681604.5	30.5	328.83
20	5078	Renould's Ground Cuckoo	unk	unk	O	Carpococcyx	renauldi	53.2	223547.5	1.15	2
21	4441	Crested Oropendola	294	male	F,O	Psarocolius	decumanus	50.08	4937430.5	23.046	37.7
Aviary Data #4 Thursday June 9th,1999											

S.NO	ISIS	COMMON NAME	BW(g)	SEX	DIET	GENUS	SPECIES				
22	150	Ring necked pheasant invalid number			G,O	Phasianus	colchicus	59.66	4618416	25.68	unk
23	3773	Common beafowl Wompoo Fruit Dove	187	male	F,O	Ptilinopus	magnificus	75.85	5544829	39.2	97.96
24	5078	Renauld's Ground Cuckoo)		unk	O	Carpococcyx	renauldi	117.94	207752	2.28	1.93
25	122	Red and white craker	42	male	O	Laterallus	leucopyrrhus	~83.64	9344294	55.87	210.03
26	5135	Chilean tinamou	273	unk	H,O	Nothoprocta	perdicaria	96.88	207623	1.87	2.03
27	4121	Elegant crested tinamou	605	male	H,O	Eudromia	elegans	78.78	1274271	9.35	45.36
28	4881	Golden-headed Tananger	17.7	unk	O	Tangara	larvata	233.95	5	5.19	5.133
29	1083	Amethyst starling	49.5	female	O	Cinnyricinclus	leucogaster	119.06	2067814	22.93	134.33
30	4766	Goldie's lorikeet	53	female	F,N,O	Trichoglossus	goldiei	61.82	1869296.5	10.77	31.26
31	4190	Blue-crowned hanging parrot	28.85	male	F,N,O	Loriculus	galgulus	*129.69	7235957	87.46	52.66
32	4594	Black-necked stilt	147	male	O	Himantopus	himantopus	*~97.9	857778.5	6	30.7
33	4737	American avocet	289		O	Recurvirostra	americana	102.13	372876.5	3.54	10.03
34	4498	White-crested Turaco	163	female	F,O	Tauraco	leucolophus	88.27	2178529.5	17.92	38
Aviary Data #5 Friday July,		July 9th, 1999									
S.NO	ISIS	COMMON NAME	BW(g)	SEX	DIET	GENUS	SPECIES				
50	6m	Racing pigeon(1yr)	429	unk	G,O	Columba	livia	91.88	898679	7.69	6.466
51	4158	Palm Tanager	38	female	O	Thraupis	palmarum	~141.2	2742471	27.68	60.53
52	5060	Crested orapendola Crested Oropendola	272	unk	F,O	Psarocolius	decumanus	89.1	967913.5	8.03	2.066
53	3325	Speckled mousebird	39	male	F,O	Colius	striatus	~66.49	5718714	27.18	119.23
54	3129	Lilac-brested Roller	108.5	male	O	Coracias	caudata	69.12	8700923	56.05	121.46
55	3644	Lilac brested roller	91	female	O	Coracias	caudata	63.83	6042586	35.94	102.26
56	4890	Black crake(5mnths)	54.2	male	O	Limnocorax	flavirostra	78.45	349429	2.55	4.766
57	5959	Racing Pigeon	287	unk	G,O	Columba	livia	~69.86	5432095	27.13	unk
58	3288	Blue-crowned Motmot	120	female	O	Momotus	momota	~95.19	4591676	31.24	140.6
59	4336	Great Egret(skin from leg)9yrs.	948	unk	P,C=O	Egretta	alba	*~144.76	12877908.5	133.27	108

60	16	Snowy Egret(very old)	418	unk	P,C=O	Egretta	thula	~55.939	21702263	86.73	unk
61	4928	Great blue heron	1800	unk	P,C=O	Ardea	herodias	~53.15	2329516.5	8.85	2.6
Aviary Data # 6 Thursday, August 5th, 1999											
S.NO	ISIS	COMMON NAME	BW(g)	SEX	DIET	GENUS	SPECIES				
62	4904	Nompoo fruit dove Wompoo Fruit Dove	200	male	F,O	Ptilinopus	Magnificus	*81.8	5110887.5	38.96	10.53
63	3062	Schalout's turaco Schalow's Turaco	187	male	F,O	Tauraco	corythaix	~54.26	8624424.5	33.45	92.9
64	5039	Sooty headed bulhi Sooty-headed Bulbul	32	male	O	Pycnonotus	aurigaster	~135.25	810330.5	7.83	60.93
65	1969	Nicobar Pigeon	545	male	F,G=O	Caloenas	nicobarica	~71.97	4902347	25.22	140.3
66	5173	African pygmy goose	170	female	O	Nettapus	auritus	96.89	466715.5	4.21	8.56
Aviary Data # 7 Thursday, October 21st, 1999			BW(g)	SEX	DIET	GENUS	SPECIES				
S.NO	ISIS	COMMON NAME									
67	5312	Chilean Tinamou	476	unk	H,O	Nothoprocta	perdicaria	~67.19	1587057.5	7.62	6.53
68	4912	Southern White-bellied Caique	161.3	Male	O	Pionites	leucogaster	~44.97	5537349	17.8	28.36
69	3233	Fire-tufted Barbet	110	female	O	Psilopogon	pyrolophus	37.61	13658776.5	47.88	129.7
70	5000	Racing Pigeon	354	unk	G,O	Columba	livia	*~43.69	9398202	29.35	18.36
71		Rock Dove(Red leg band)No ID	unk	unk	G,O	Columba	livia	119.75	1341167	14.96	unk
72	4765	Golden Lorikeet Goldie's Lorikeet	45	female	F,N=O	Trichoglossus	goldiei	48.84	2081530	17.76	6.33
73	5264	Pheasant Pigeon	416	female	O	Otidiphaps	nobilis	~44.59	1214680	3.87	96.83
74	4394	Pheasant Pigeon	469	female	O	Otidiphaps	nobilis	71.7	3507042	23.44	65.3
75	3321	Hooded Pitta	45	female	O	Pitta	sordida	153.46	2807266	40.15	134.3

Figure 4. Pentosidine standard curve used to calculate pentosidine concentrations in the foot webbing of California Gulls.



Standards

pmol Ps	Avg Area
14.5	4099808
7.25	2048371
3.625	1014487
1.8125	501895
0.90625	245800

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